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Report

Neonatal and Fetal Methylenetetrahydrofolate Reductase Genetic Polymorphisms: An Examination of C677T and A1298C Mutations

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Methylenetetrahydrofolate reductase (MTHFR) mutations are commonly associated with hyperhomocysteinemia, and, through their defects in homocysteine metabolism, they have been implicated as risk factors for neural tube defects and unexplained, recurrent embryo losses in early pregnancy. Folate sufficiency is thought to play an integral role in the phenotypic expression of MTHFR mutations. Samples of neonatal cord blood (n = 119) and fetal tissue (n = 161) were analyzed for MTHFR C677T and A1298C mutations to determine whether certain MTHFR genotype combinations were associated with decreased in utero viability. Mutation analysis revealed that all possible MTHFR genotype combinations were represented in the fetal group, demonstrating that *677T* and *1298C* alleles could occur in both *cis* and *trans* configurations. Combined *677CT/1298CC* and *677TT/1298CC* genotypes, which contain three and four mutant alleles, respectively, were not observed in the neonatal group (P = .0402). This suggests decreased viability among fetuses carrying these mutations and a possible selection disadvantage among fetuses with increased numbers of mutant MTHFR alleles. This is the first report that describes the existence of human *MTHFR 677CT/1298CC* and *677TT/1298CC* genotypes and demonstrates their potential role in compromised fetal viability.

Methylenetetrahydrofolate reductase (MTHFR; E.C. 1.5.1.20 [MIM 236250]) is essential to homocysteine metabolism (Frosst et al. 1995). Mutations in MTHFR and other genes associated with homocysteine metabolism have been reported as causes of hyperhomocysteinemia (Frosst et al. 1995; Jacques et al. 1996). MTHFR mutations, however, are by far the most common etiology of the mild-to-moderate form of hyperhomocysteinemia that is secondary to inherited enzymatic defects, whereas folate deficiency remains the primary cause of hyperhomocysteinemia (Isotalo and Donnelly 2000).

The best-characterized MTHFR genetic polymorphism is a common missense mutation consisting of a 677C→T transition (Frosst et al. 1995). This mutation produces an alanine to valine amino acid substitution within the predicted catalytic domain of the MTHFR enzyme (Frosst et al. 1995; van der Put et al. 1998). The result is a thermolabile enzyme variant that has reduced catalytic activity. Folate acts to stabilize this thermolabile enzyme (Frosst et al. 1995; Rozen 1997). Homozygosity for the thermolabile MTHFR variant predisposes individuals to the development of hyperhomocysteinemia, especially during times of folate insufficiency (Frosst et al. 1995; van der Put et al. 1998). Serum folate values >15.4 nM appear to neutralize the effects of 677C→T mutations (Jacques et al. 1996).

Another common genetic polymorphism of MTHFR is a missense mutation, consisting of a nucleotide 1298A→C transition, that results in a glutamate to alanine substitution (van der Put et al. 1998). Unlike the 677T mutation, the 1298C mutation is located in the presumed regulatory domain of MTHFR (van der Put et al. 1998; Weisberg et al. 1998). In addition, the *1298C* mutation, in either the heterozygous or the homozygous state, does not appear to cause elevations in plasma homocysteine. However, combined heterozygosity for both 677T and 1298C mutations, which produces a *677CT/1298AC* genotype, does result in significant

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plasma homocysteine elevations (van der Put et al. 1998).

Neural tube defects (NTDs), stillbirths, and recurrent abortions have all been associated with hyperhomocysteinemia (Wouters et al. 1993; Mills et al. 1995). Through their defects in homocysteine metabolism, MTHFR mutations have also been implicated as risk factors for NTDs (van der Put et al 1995, 1998; Whitehead et al. 1995; Christensen et al. 1999; Shields et al. 1999) and unexplained, recurrent embryo losses in early pregnancy (Nelen et al. 1997). Because most NTD-risk studies that have investigated the role of MTHFR mutations have focused on living children and parents, intrauterine fetal demise secondary to MTHFR mutations cannot be dismissed (Christensen et al. 1999). We have previously shown that these mutations can occur in *cis* configurations; however, we have not observed *677T* alleles in the presence of homozygosity for the *1298C* allele (Isotalo and Donnelly 2000). To investigate the relationship between MTHFR mutations and fetal viability, we examined the prevalence of both neonatal and fetal MTHFR C677T and A1298C mutations, to determine whether certain MTHFR genotype combinations were underrepresented in either group.

All procedures were performed in accordance with the guidelines of the institutional review board of the Ottawa Hospital and with appropriate informed consent. The study group consisted of 161 fetal tissue samples from both spontaneous and therapeutic terminations of pregnancy. This study group was consecutively selected; however, the method of case recruitment precluded the inclusion of embryos lost during very early pregnancy. Sex distribution and the presence or absence of NTDs could not be determined in this group. For the control group, 119 neonates were randomly selected, and umbilical cord–blood samples were obtained from each. No exclusion criteria existed for the control group, which was composed of samples from 57 female and 62 male infants. No cases of NTDs or homocysteinuria occurred in the control group.

Genomic DNA was extracted from formalin-fixed fetal tissue and neonatal cord whole blood, by use of the Promega Wizard genomic–DNA extraction kit (Promega) according to the manufacturer's protocol. To detect MTHFR 677C→T and 1298A→C mutations, PCR amplification and RFLP analysis were performed according to published techniques (Donnelly and Rock 1999; Isotalo and Donnelly 2000). The 677C→T mutation introduces a new *Hin*fI restriction site that results in digestion of a 198-bp PCR amplicon into 175- and 23-bp fragments. The 1298A→C mutation abolishes an *Mbo*II restriction site resulting in a 163-bp amplicon that is digested into four fragments of 84, 31, 30, and 18 bp. The wild-type *1298AA* genotype yields an amplicon digest of five fragments of 56, 31, 30, 28, and 18 bp. Digested PCR fragments were separated electrophoretically, by use of 1% agarose (Gibco-BRL) and 2.5% Metaphor™ agarose gels (FMC Bioproducts) prepared with 1 × Tris-borate EDTA (Sigma) and ethidium bromide (Sigma).

MTHFR allele frequencies and the prevalences of single and combined MTHFR genotypes were determined for the study group and the control group and were compared by a two-tailed Fisher's exact test. Statistical significance was defined as $P < .05$. Statistical analysis was performed using GraphPad InStat™ (GraphPad Software).

Individual MTHFR genotype distributions for neonatal and fetal groups are presented in table 1. The *677TT* genotype was more prevalent in the neonatal group at 11.8% than in the fetal group (3.1%) ($P = .0068$). No individual MTHFR genotypes, other than the *677TT* genotype, showed statistically significant differences in distribution between groups. Combined MTHFR C677T/A1298C genotype distributions for both groups are presented in table 2, along with the frequencies of single MTHFR alleles. MTHFR mutations were common. The *677T* allele frequency was .217 and .273 for the fetal and neonatal groups, respectively, whereas the *1298C* allele frequency was .401 and .357, respectively, for the same groups. Analysis of combined MTHFR genotypes revealed an increased prevalence of *677TT/1298AA* genotypes in the neonatal group compared with the fetal group ($P = .001$). In addition, combined *677CT/1298CC* and *677TT/1298CC* genotypes, which correspond to triple- and quadruple-mutation combinations, respectively, were not observed in the neonatal group but were observed at low frequencies in the fetal group ($P = .0402$). All possible combined MTHFR genotypes were represented in the fetal group. Within the neonatal group, there was no significant sex-distribution difference (data not shown) between the single and the combined MTHFR genotypes.

Combined *MTHFR 677TT/1298AC* genotypes were identified at low frequencies in both the fetal and the

Table 1

Individual MTHFR Genotype Distributions for Neonatal and Fetal Groups

MTHFR Genotype	Observed Frequency		P[a]
	Neonatal Group ($n = 119$)	Fetal Group ($n = 161$)	
677CC	.571	.596	.714
677CT	.311	.373	.311
677TT	.118	.031	.007
1298AA	.361	.342	.800
1298AC	.563	.515	.468
1298CC	.076	.143	.090

[a] By Fisher's exact test.

Table 2

Combined C677T/A1298C MTHFR Genotype Frequencies and Allele Frequencies for Neonatal and Fetal Groups

Genotype or Allele	Observed Frequency		Odds Ratio (95% CI)	P^b
	Neonatal Group (n = 119)[a]	Fetal Group (n = 161)		
MTHFR C677T/A1298C genotype:				
CC/AA	.143	.155	1.1 (.57–2.2)	.866
CC/AC	.353	.335	.93 (.6–1.5)	.800
CC/CC	.076	.107	1.3 (.6–3.2)	.415
CT/AA	.117	.174	.2 (.8–3.2)	.237
CT/AC	.193	.168	.8 (.5–1.6)	.637
CT/CC	NO	.031	8.4 (.5–153.5)	.074
TT/AA	.101	.012	.1 (.02–.5)	.001
TT/AC	.017	.012	.7 (.1–5.3)	1.000
TT/CC	NO	.006	2.2 (.1–55.4)	1.000
Combined CT/CC and TT/CC	NO	.037	10.0 (.6–179.2)	.040
MTHFR allele:				
677C	.727	.783		
677T	.273	.217		
1298A	.643	.599		
1298C	.357	.401		

[a] NO = not observed.
[b] By Fisher's exact test.

neonatal groups and demonstrated that MTHFR mutations could occur in *cis* configurations. This provides evidence that crossover of MTHFR 677C→T and 1298A→C mutations has occurred in the study population.

The regulation of homocysteine metabolism is complex and is dependent on multiple vitamin cofactors, including folate, pyridoxine and vitamin B_{12} (Jacques et al. 1996). Both vitamin-cofactor deficiencies and enzyme deficiencies may contribute to hyperhomocysteinemia. Folate deficiency is the leading cause of hyperhomocysteinemia; however, MTHFR mutations also affect folate metabolism, resulting in increased homocysteine.

Both hyperhomocysteinemia and MTHFR mutations have been associated with NTDs (van der Put et al. 1995, 1998; Whitehead et al. 1995; Christensen et al. 1999; Shields et al. 1999). Folate sufficiency is believed to play a critical role in the phenotypic expression of these mutations. Outright folate deficiency is uncommon in women who give birth to neonates with NTDs; therefore, periconceptional folate therapy does more than correct a nutritional deficiency (Shields et al. 1999). One of its effects is probably to stabilize MTHFR mutations and, thereby, to reduce the severe phenotypic expression of these mutations. In addition to NTDs, decreased fetal viability secondary to MTHFR mutations has been reported by Nelen et al. (1997), who demonstrated that maternal homozygosity for the *677T* mutation was associated with a twofold- to threefold-increased risk for recurrent loss of an embryo during early pregnancy. In their examination of 18 fetuses with anencephaly, encephalocele, and/or spina bifida, Stegmann et al. (1999) found no significant deviation in MTHFR genotype distribution in affected fetuses and controls. They concluded that extreme forms of NTDs, those more likely to be associated with compromised fetal viability, are unlikely to be the result of MTHFR polymorphisms. We provide evidence to suggest that combined *MTHFR 677T* and *1298C* mutations may be responsible for compromised fetal viability.

Examination of combined genotypes revealed that all possible MTHFR-allele combinations were represented in the fetal group. In the neonatal group, both the *MTHFR 677CT/1298CC* and the *677TT/1298CC* genotypes were absent. Combined, these genotypes exhibited a statistically significant distribution difference, compared with what is seen in the fetal group (P = .0402). We have also observed the absence of *677CT/1298CC* and *677TT/1298CC* genotypes in an adult population consisting of healthy controls and patients with venous thrombosis (n = 129) (Isotalo and Donnelly 2000). The *677CT/1298CC* and *677TT/1298CC* combined genotypes are significant, because they carry three and four mutant MTHFR alleles, respectively. The absence of quadruple and certain triple MTHFR-mutation combinations, in both neonates and adults (Isotalo and Donnelly 2000), may be secondary to compromised fetal viability, because *677T* and *1298C* mutations are known to interact in vivo (van der Put et al. 1998; Weisberg et al. 1998). It is interesting to note that *MTHFR 677TT/1298AC* genotypes, which also contain three mutant alleles, were represented in both groups, indicating a po-

tential in vivo enzymatic-activity difference between the *677CT/1298CC* genotype and the *677TT/1298AC* genotype. It is unknown how the polymorphisms at positions *677* and *1298* interact with each other. MTHFR is a dimeric protein that increases the number of phenotypic forms that can be predicted for certain genotypes. Although there is no evidence that the dimeric form of this protein shows cooperativity between the two subunits, it is likely that certain phenotypes, particularly those which arise from compound heterozygotes, have either decreased stability or altered catalytic activity as is the case when only the *677T* allele is considered.

The *677TT* genotype was observed less frequently in fetuses than in neonates ($P = .0068$) and was also accompanied by a nearly 10-fold increase in frequency of *677TT/1298AA* combined genotypes in the neonatal group, compared with the fetal group ($P = .001$). The *MTHFR 677TT/1298CC* genotype was also uncommon in the fetal group, although the *677CT/1298CC* genotype was more prevalent. Because both these genotypes appear to carry a selection disadvantage and to lead to compromised fetal viability, there would be proportionally greater removal of *677CT* genotypes than of *677TT* genotypes. This selection effect could explain the increased *677TT* and *677TT/1298AA* genotype representation in our neonatal group.

Complete linkage disequilibrium between the *MTHFR 677T* and *1298C* mutations has been suggested, because the majority of studies examining these mutations have identified them in *trans* positions only (van der Put et al. 1998; Rady et al. 1999; Stegmann et al. 1999). Weisberg et al. (1998) have identified one child with an NTD who had an *MTHFR 677TT/1298AC* genotype, providing evidence that *cis* configurations of MTHFR mutations do occur. In a previous study of MTHFR mutation prevalence in patients with venous thrombosis and in a control group, we identified a *677TT/1298AC* genotype frequency of 9.2% and 12.5%, respectively, for these groups. This finding clearly indicates that, if linkage disequilibrium between MTHFR mutations is present, it is incomplete (Isotalo and Donnelly 2000). In spite of the short physical distance (2.1 kb) between MTHFR mutation loci, we have observed evidence of recombinant events between MTHFR mutations in both the fetal and the neonatal groups. The identification of *cis* MTHFR mutations is significant, because it allows more than two mutant alleles to be present in the genome of a fetus. van der Put et al. (1998) theorized that, if *cis* configurations of MTHFR mutations occurred, they would result in a selection disadvantage because of the expression of severe phenotypes, a category that could include both NTDs and spontaneous abortions. The identification of *677CT/1298CC* and *677TT/1298CC* genotypes in the fetal group provides further evidence for the *cis* configuration of MTHFR mutations. The absence of these same genotype combinations in the neonatal group suggests that additional MTHFR mutations in *cis* are potentially deleterious or lethal. This is the first report that describes the existence of human *MTHFR 677CT/1298CC* and *677TT/1298CC* genotypes and that demonstrates their potential role in compromised fetal viability.

The inherent limitation of our study (and others that have examined the role of MTHFR mutations in contributing to NTDs and/or decreased fetal viability) is that there is bias toward late-pregnancy outcomes. Because the majority of spontaneous abortions occur before pregnancy is even identified clinically, the true contribution of MTHFR mutations to fetal viability may be difficult to determine. Larger prospective studies of both viable and nonviable fetuses, along with multigenerational genotyping, may further elucidate the relationship between MTHFR genotypes and clinical outcomes.

In summary, combined MTHFR mutations likely carry a selective disadvantage and contribute to decreased fetal viability, especially during times of folate insufficiency. *MTHFR 677T* and *1298C* mutations, despite the short physical distance between their loci, have shown evidence of allele crossover in our study population. This study provides evidence that the combined effects of multiple mutations on phenotype, particularly in the case of common polymorphisms, are not restricted to intergenic interactions. The recognition that combined MTHFR genotypes may influence pregnancy outcomes further demonstrates both the clinical significance of this gene and the potential protective role of folate sufficiency.

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Electronic-Database Information

The accession number and URL for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for MTHFR [MIM 236250])

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